

Biotransformation of the Major Fungal Metabolite 3,5-Dichloro-*p*-Anisyl Alcohol under Anaerobic Conditions and Its Role in Formation of Bis(3,5-Dichloro-4-Hydroxyphenyl)methane

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Higher fungi have a widespread capacity for biosynthesis of organohalogenes. Commonly occurring chloro-aromatic fungal metabolites can end up in anaerobic microniches at the boundary of fungal colonies and wetland soils. The aim of this study was to investigate the environmental fate of a major fungal metabolite, 3,5-dichloro-*p*-anisyl alcohol, under anaerobic conditions. This compound was incubated with methanogenic sludge to study its biotransformation reactions. Initially, 3,5-dichloro-*p*-anisyl alcohol was readily demethylated in stoichiometric quantities to 3,5-dichloro-4-hydroxybenzyl alcohol. The demethylated product was converted further via two routes: a biotic route leading to the formation of 3,5-dichloro-4-hydroxybenzoate and 2,6-dichlorophenol, as well as an abiotic route leading to the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane. In the first route, the benzyl alcohol moiety on the aromatic ring was oxidized, giving 3,5-dichloro-4-hydroxybenzoate as a transient or accumulating product, depending on the type of methanogenic sludge used. In sludge previously adapted to low-molecular-weight lignin from straw, a part of the 3,5-dichloro-4-hydroxybenzoate was decarboxylated, yielding detectable levels of 2,6-dichlorophenol. In the second route, 3,5-dichloro-4-hydroxybenzyl alcohol dimerized, leading to the formation of a tetrachlorinated bisphenolic compound, which was identified as bis(3,5-dichloro-4-hydroxyphenyl)methane. Since formation of this dimer was also observed in incubations with autoclaved sludge spiked with 3,5-dichloro-4-hydroxybenzyl alcohol, it was concluded that its formation was due to an abiotic process. However, demethylation of the fungal metabolite by biological processes was a prerequisite for dimerization. The most probable reaction mechanism leading to the formation of the tetrachlorinated dimer in the absence of oxygen is presented, and the possible environmental implications of its natural occurrence are discussed.

Organohalogenes are usually perceived by the public as undesirable pollutants of anthropogenic origin. However, 2,450 different naturally occurring halogenated compounds have been identified so far (15). The higher fungi, basidiomycetes, have a widespread capacity for biosynthesis of organohalogenes (39). Adsorbable organic halogens (AOX) and/or low-molecular-weight halogenated compounds are produced by 68 genera of basidiomycetes from 20 different families (11). Most of the 81 halogenated metabolites identified from basidiomycetes to date are chlorinated, although brominated and iodated metabolites have also been demonstrated.

The compound 3,5-dichloro-*p*-anisyl alcohol belongs to the group of chlorinated anisyl metabolites (CAM) and is a major metabolite of fungi belonging to the genera *Hypholoma*, *Pholiota*, *Stropharia*, *Lepista*, *Oudemansiella*, *Phellinus*, *Phylloporia*, and *Bjerkandera* (11). When species belonging to these genera were grown in liquid culture media, the concentrations of 3,5-dichloro-*p*-anisyl alcohol ranged from 2.4 mg/liter in cultures of *Phellinus torulosus* to 108.4 mg/liter in cultures of *Hypholoma elongatum* (37).

H. elongatum (Pers. ex Fr.) Ricken is a fungal species typical of wetlands, where it grows in moss (*Sphagnum* spp. and *Polypodium* spp.). The fungus is rather general in The Netherlands,

since it appears in 10 to 25% of the 5-km²-grid blocks investigated (3). *Hypholoma fasciculare* is the most commonly occurring species of the basidiomycetes in The Netherlands, since it was observed in 69% of the 5-km²-grid blocks investigated and showed the highest percentage (1.3%) of all mushroom sightings (30). The 3,5-dichloro-*p*-anisyl alcohol concentrations of this fungus were up to 36.2 and 71.2 mg per liter in nitrogen-limited and nitrogen-rich culture media, respectively (40). When cultivated on forest litter, concentrations of 3,5-dichloro-*p*-anisyl alcohol reached 204.9 mg per kg (dry weight) of substrate after 84 days of incubation.

Direct measurements in the environment have also demonstrated that 3,5-dichloro-*p*-anisyl alcohol is an important fungal compound. The concentrations of 3,5-dichloro-*p*-anisyl alcohol and 3,5-dichloro-*p*-anisaldehyde in wood samples colonized by *Hypholoma* spp. at forested sites ranged from 24 to 180 mg of CAM per kg (dry weight) of sample (10). Estimations showed that the yearly production of AOX by *H. fasciculare* was 110 g of AOX per hectare of forest (40). Most of the AOX is due to CAM compounds.

In wetlands with *H. elongatum* and wet forest soils with *H. fasciculare*, 3,5-dichloro-*p*-anisyl alcohol can end up in anaerobic microniches at the boundary of the fungal colonies and soil. Except for the studies examining the anaerobic biodegradability of 2,4-dibromophenol and other brominated phenols produced by marine hemichordates (20, 35), nothing is known about the environmental fate of natural organohalogenes under anaerobic conditions. The purpose of this study was to study the environmental fate of the major fungal metabolite 3,5-

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dichloro-*p*-anisyl alcohol under anaerobic conditions. This was done by incubating the compound with methanogenic sludge, which was cultivated on either straw extracts or volatile fatty acids, and studying its biotransformation reactions.

MATERIALS AND METHODS

Methanogenic sludge and culture conditions. Granular methanogenic sludge, previously cultivated on alkaline extracts of wheat straw (WS-sludge), was obtained from S. Kortekaas, Department of Environmental Technology, Wageningen Agricultural University, Wageningen, The Netherlands. WS-sludge was grown for more than 1 year in a 1-liter upward flow anaerobic sludge bed (UASB) reactor fed with a filtered (cheesecloth) black liquor extract diluted to 8 g of chemical oxygen demand (COD) per liter. The load of the reactor was 23 g of COD per liter per day. The black liquor extract was prepared from 100 g of wheat straw and 10 g of Na_2CO_3 in 1 liter of tap water (120°C for 2 h). The diluted black liquor extract was supplemented as follows: yeast extract, 0.1 g/liter (Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom); NH_4Cl , 0.28 g/liter; KH_2PO_4 , 0.25 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/liter; CaCl_2 , 0.008 g/liter; trace elements solution, 1 ml; and the antifoam agent Klaraid 4039, 0.05 ml (Grace Dearborn, Mijdrecht, The Netherlands). The composition of the trace elements solution was described by Zehnder et al. (43). WS-sludge was washed with tap water and centrifuged ($30,000 \times g$, 15 min) before use. One gram of fresh WS-sludge contained 0.0919 g of volatile suspended solids (VSS). The ash content of the dry sludge solids was 8.1%.

Granular methanogenic sludge, previously grown on volatile fatty acids (VFA-sludge), was obtained from M. van Eckert, Department of Environmental Technology, Wageningen Agricultural University, Wageningen, The Netherlands. VFA-sludge was grown for more than 3 years in a 10-liter UASB reactor on a mixture of sodium acetate (18.6 mM), sodium propionate (14.2 mM), and sodium butyrate (13 mM), supplemented with minerals and trace elements according to the method of Alphenaar (1). VFA-sludge was washed with tap water and centrifuged ($30,000 \times g$, 15 min) before use. One gram of fresh VFA-sludge contained 0.1587 g of VSS, and the ash content of the dry solids was 20.6%.

Biotransformation experiments. 3,5-Dichloro-*p*-anisyl alcohol was used as a model substrate. The composition of the medium was as follows: 3,5-dichloro-*p*-anisyl alcohol, 0.35 g/liter; NaHCO_3 , 2.50 g/liter; $(\text{NH}_4)_2\text{HPO}_4$, 0.07 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g/liter; and sodium acetate, 0.25 g/liter. The pH was adjusted to 7.2. Medium (25 ml in 100-ml serum bottles) was inoculated with 0.25 g of wet WS-sludge or VFA-sludge and stoppered with *n*-butyl stoppers. Bottles were made anaerobic by flushing with 70/30 N_2 - CO_2 and were incubated on a rotary shaker (100 rpm) at 30°C in the dark. Bottles with substrate but lacking sludge and with sludge but lacking 3,5-dichloro-*p*-anisyl alcohol were incubated in parallel to the biotransformation bottles and were harvested as controls. The biotransformation experiment was conducted in triplicate. Samples were taken daily. At the time of sampling, separately incubated bottles were sacrificed for analysis.

Formation of bis(3,5-dichloro-4-hydroxyphenyl)methane. (i) **Experiment I.** In order to study the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane, 3,5-dichloro-4-hydroxybenzyl alcohol (0.1 g/liter) was incubated with deuterated 2,6-dichlorophenol. Otherwise the composition of the medium was identical to that described for the biotransformation experiments. The incubations of 100 ml of medium in 250-ml serum bottles were inoculated with 1.00 g of wet WS-sludge, stoppered, flushed, and incubated as referred to above. Two times per week, a concentrated solution of deuterated 2,6-dichlorophenol was added to the incubations, providing a final concentration of 5 mg per liter. Triplicate incubated bottles were sacrificed for analysis two times per week and analyzed for concentrations of 3,5-dichloro-4-hydroxybenzyl alcohol, deuterated 2,6-dichlorophenol, and adduct with and without incorporated deuterium label.

(ii) **Experiment II.** In a subsequent experiment, 3,5-dichloro-*p*-anisyl alcohol (0.25 g per liter) and 3,5-dichloro-4-hydroxybenzyl alcohol (0.25 g per liter) were separately incubated with living and autoclaved WS-sludge under anaerobic and aerobic conditions as well as sequential anaerobic-aerobic conditions. The composition of the medium was the same as that in the biotransformation experiments. The medium (100 ml in 250-ml serum bottles) was inoculated with 1.00 g of wet WS-sludge, stoppered with *n*-butyl stoppers, and made anaerobic by flushing with 70/30 N_2 - CO_2 . Half of the bottles were autoclaved two times on subsequent days for 1 h at 121°C. All bottles were incubated on a rotary shaker (100 rpm) at 30°C in the dark. The bottles that were incubated aerobically were injected with 50 ml of pure O_2 . Control bottles with test compound but lacking sludge were incubated in parallel and were harvested as controls. After 10 days of incubation, half of the bottles initially incubated anaerobically with living and autoclaved sludge were aerated by injection of 50 ml of pure O_2 per bottle, after which, bottles were incubated again for 6 weeks. At the time of sampling, bottles were sacrificed in duplicate for analyses of all chlorinated compounds present.

Extraction procedure and compound identification. Medium with sludge was filtered over a paper filter (type V259; Schut BV, Heelsum, The Netherlands), after which the filtrate and the filter with the sludge were separated. The filtrate, after adjustment to pH 2 with 4 M H_2SO_4 , was extracted three times with 10 ml of freshly distilled ethyl acetate, and organic layers were combined. A filter with sludge was subjected to overnight soxhlet extraction with freshly distilled ethyl

acetate. The filtrate and the filter-sludge extracts were washed with water and concentrated under reduced pressure at ambient temperature. The concentrate was filtered over silica gel 60 (230 to 400 mesh; Merck, Darmstadt, Germany) with ethyl acetate as the eluent. After removal of the solvent under reduced pressure, the residue was redissolved in 1 ml of ethyl acetate containing 410 μg of 4-bromoanisole as the internal standard. The substrate and derived compounds were identified by gas chromatography (GC) analyses and, incidentally, GC-mass spectrometry (MS). GC analyses were performed with a Varian 3600 gas chromatograph equipped with a fused-silica capillary column (DB17; 30 m by 0.25-mm internal diameter film thickness, 0.25 μm) and a 1:1 end splitter with each split leading to separate detectors. Parallel detection was carried out by flame ionization detection (FID) and electron capture detection (ECD). The carrier gas and flow were N_2 at 1.2 ml per min. The injector temperature was 220°C, the FID temperature was 230°C, the ECD temperature was 275°C, and the temperature program was 70 to 250°C at 7°C per min and then hold for 20 min. The injection volume was 10 μl . The split ratio was 1:100. GC-MS analyses were performed on a HP5970B quadrupole mass spectrometer coupled to a HP5890 gas chromatograph equipped with a fused-silica capillary column (DB17; internal diameter; 30 m by 0.25-mm film thickness, 0.25 μm). The carrier gas and flow were He at 1.1 ml per min. The injector temperature was 220°C. The temperature program was identical to that used for GC. The injection volume was 10 μl , and the split ratio was 1:100. Electron impact-MS data were obtained at 70 eV. Identification of compounds was achieved by comparison of retention times and mass spectra with data of synthetic reference compounds.

Reference compounds. 3,5-Dichloro-*p*-anisyl alcohol was prepared as described by de Jong et al. (10). 3,5-Dichloro-4-hydroxybenzyl alcohol was prepared by reduction of methyl 3,5-dichloro-4-hydroxybenzoate (Lancaster, Mülheim am Main, Germany) with LiAlH_4 in ether. 3,5-Dichloro-4-hydroxybenzoic acid was purchased from Lancaster. 2,6-Dichlorophenol was purchased from Aldrich (Aldrich-Chemie, Steinheim, Germany). 2,6-Dichlorophenol-*d*₂ was prepared by carboxylation commercially available (Acros Organics, Geel, Belgium) deuterated phenol-*d*₅ in the *para* position according to Komiya and Hirai (21). The obtained deuterated 4-hydroxy benzoate was chlorinated and subsequently decarboxylated, yielding 2,6-dichlorophenol-*d*₂ as a white solid (38).

The adduct bis(3,5-dichloro-4-hydroxyphenyl)methane was prepared by purging a solution of 1.0 g (5.0 mmol) of bis(4-hydroxyphenyl)methane in 30 ml of acetic acid with Cl_2 at room temperature. The mixture, which contained a white precipitate, was stirred for 30 min and then filtered. The residue was recrystallized from acetic acid to give 0.65 g (39%) of the adduct as a white solid. The melting point, was 194 to 195°C (184 to 185°C [6]). For ^1H NMR (200 MHz, acetone-*d*₆), δ (ppm) was as follows: 3.85 (s, 2H, CH_2) and 7.26 (s, 4H, C-2, C-2', C-6 and C-6'). For ^{13}C NMR (acetone-*d*₆), δ (ppm) was as follows: 39.2 (t, CH_2), 122.6 (4s, C-3, C-3', C-5 and C-5'), 129.6 (4d, C-2, C-2', C-6, and C-6'), 134.9 (2s, C-1 and C-1'), and 148.4 (2s, C-4 and C-4'). For high-resolution MS, the value calculated for $\text{C}_{13}\text{H}_8\text{Cl}_4\text{O}_2$ (M^+) was 335.9278; that found was 335.9278. For MS, *m/e* (relative intensities) were as follows [$\text{M} + 4$]⁺ (28); [$\text{M} + 2$]⁺ (58); and [M]⁺ (46); 301 (100); 265 (16); 231 (37); 202 (17); 175 (29); 139 (23); 115 (26); 101 (41); 87 (13); and 75 (25).

RESULTS

Biotransformation experiment. Biotransformation of 3,5-dichloro-*p*-anisyl alcohol by WS-sludge started after 1 day of incubation (Fig. 1A). Initially, the methoxy-group was demethylated quantitatively, yielding 3,5-dichloro-4-hydroxybenzyl alcohol as a product after 6 days of incubation. Thereafter, the benzyl alcohol group was slowly oxidized, yielding 3,5-dichloro-4-hydroxybenzoic acid as a product. After 20 days of incubation, most of the 3,5-dichloro-4-hydroxybenzyl alcohol formed was eliminated. Part of the 3,5-dichloro-4-hydroxybenzoic acid formed was subsequently decarboxylated, resulting in the formation of 2,6-dichlorophenol as a detectable product (Fig. 1B). The final concentration of 2,6-dichlorophenol after 20 days of incubation amounted to 7.5 mg/liter. A part of the initial substrate is unaccounted for as metabolites produced at the end of incubation, which might be due to minor metabolites not detected by the analytical procedures applied. Moreover, further in this section, the occurrence of a new tetrachlorinated bisphenolic compound in the incubation bottles will be reported.

In the biotransformation experiments with VFA-sludge, the conversions of 3,5-dichloro-*p*-anisyl alcohol were similar to those found with the WS-sludge (Fig. 2). However, some differences were observed. Demethylation started after 7 days and was completed after 13 days of incubation. Oxidation of the benzyl alcohol group was slower with the VFA-sludge than

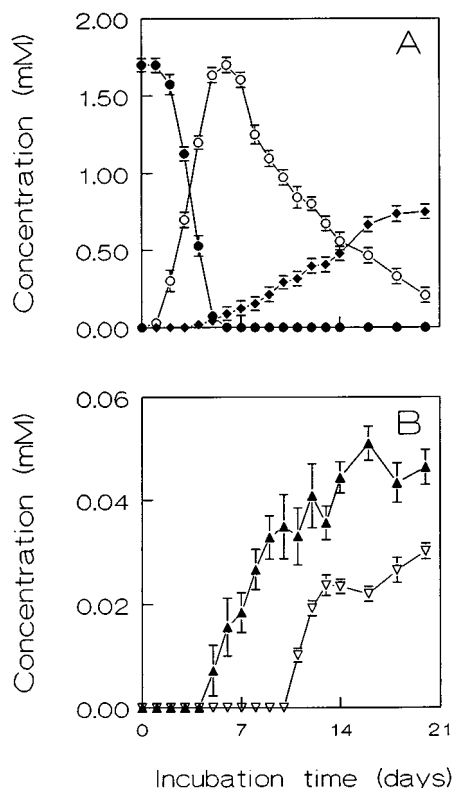


FIG. 1. Biotransformation of 3,5-dichloro-*p*-anisyl alcohol by WS-sludge under anaerobic conditions. (A) 3,5-Dichloro-*p*-anisyl alcohol (●), 3,5-dichloro-4-hydroxybenzyl alcohol (○), and 3,5-dichloro-4-hydroxybenzoic acid (◆). (B) 2,6-Dichlorophenol (▲) and bis(3,5-dichloro-4-hydroxyphenyl)methane in the aqueous phase (▽).

with WS-sludge. After 38 days of incubation, 49% of the 3,5-dichloro-4-hydroxybenzyl alcohol formed was still present in the incubation medium. In contrast to the incubations with WS-sludge, very little accumulation of 3,5-dichloro-4-hydroxybenzoic acid was observed in the incubations with the VFA-sludge. Concentrations of 3,5-dichloro-4-hydroxybenzoic acid were constant after 2 weeks of incubation and amounted to approximately 9 mg/liter. Also in contrast to the incubations with the WS-sludge, the occurrence of 2,6-dichlorophenol was not detected in the incubations with VFA-sludge during the entire experiment (Fig. 2B).

Formation of bis(3,5-dichloro-4-hydroxyphenyl)methane. During the biotransformation experiments with both sludges, a new tetrachlorinated bisphenolic compound was observed in the aqueous phase, which was identified as bis(3,5-dichloro-4-hydroxyphenyl)methane (Fig. 1B and 2B). The MS spectrum of this compound is given in Fig. 3. However, most of the compound was found to be adsorbed to the sludge and was isolated by means of soxhlet extraction with freshly distilled ethyl-acetate. The distributions of the tetrachlorinated compound over the aqueous and solid phases towards the end of the incubations were comparable between the two sludges used and were as follows: WS-sludge incubations, solid phase, 84.9 ± 3.1 mg/liter; aqueous phase, 12.0 ± 2.7 mg/liter; and VFA-sludge incubations, solid phase, 78.9 ± 2.5 mg/liter; aqueous phase, 13.8 ± 1.8 mg/liter.

In order to study the formation of the tetrachlorinated compound in more detail, 3,5-dichloro-4-hydroxybenzyl alcohol was incubated with 2,6-dichlorophenol- d_2 in the presence of WS-sludge. Incubations containing autoclaved WS-sludge were

treated similarly to the bottles with living sludge and were incubated in parallel as controls. After 17 days of incubation, the total amounts of bis(3,5-dichloro-4-hydroxyphenyl)methane in the aqueous and solid phases were 6.7 and 50.4 mg/liter in the living and autoclaved sludge incubations, respectively. The bis(3,5-dichloro-4-hydroxyphenyl)methane formed did not contain the deuterium label (Fig. 3), indicating that 2,6-dichlorophenol was not a building block of the tetrachlorinated compound. Moreover, deuterated 2,6-dichlorophenol was recovered quantitatively. This result also indicated that the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane was due to an abiotic process.

In a subsequent experiment, 3,5-dichloro-*p*-anisyl alcohol and 3,5-dichloro-4-hydroxybenzyl alcohol were incubated with living and autoclaved WS-sludge under anaerobic, aerobic, and successive anaerobic (first 10 days) and aerobic (6 weeks) conditions. The results of these incubations are given in Table 1. The bis(3,5-dichloro-4-hydroxyphenyl)methane was formed under all incubation conditions with 3,5-dichloro-4-hydroxybenzyl alcohol as the substrate, including both living and autoclaved sludge. On the other hand, if the fungal metabolite 3,5-dichloro-*p*-anisyl alcohol was used directly, the tetrachlorinated compound was only formed in the incubations with living sludges, which were incubated anaerobically. With 3,5-dichloro-4-hydroxybenzyl alcohol as the substrate, the yields of bis(3,5-dichloro-4-hydroxyphenyl)methane were higher if the sludge was autoclaved and if oxygen was present. With 3,5-dichloro-*p*-anisyl alcohol as the substrate, the succession of anaerobic and aerobic incubation conditions provided a higher

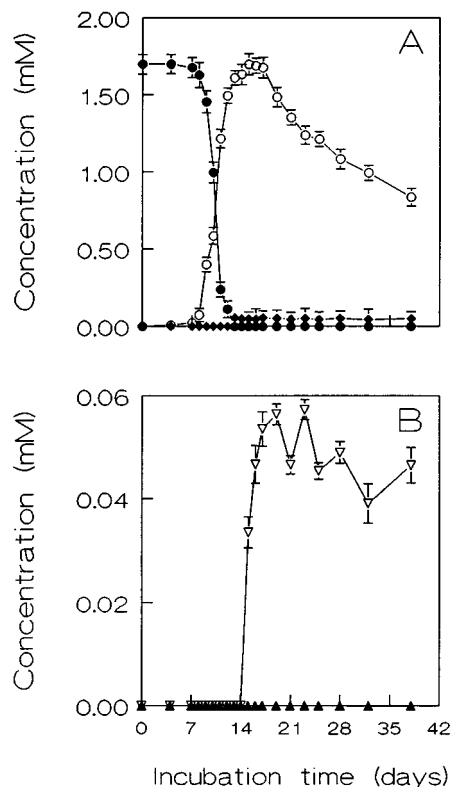


FIG. 2. Biotransformation of 3,5-dichloro-*p*-anisyl alcohol by VFA-grown methanogenic sludge under anaerobic conditions. (A) 3,5-Dichloro-*p*-anisyl alcohol (●), 3,5-dichloro-4-hydroxybenzyl alcohol (○), and 3,5-dichloro-4-hydroxybenzoic acid (◆). (B) 2,6-Dichlorophenol (▲) and bis(3,5-dichloro-4-hydroxyphenyl)methane in the aqueous phase (▽).

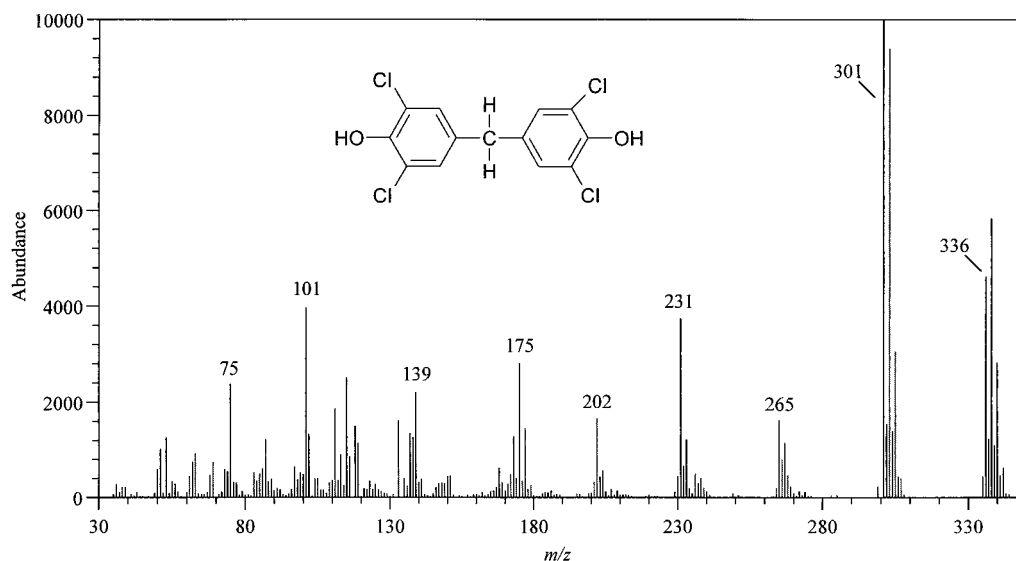


FIG. 3. MS spectrum of bis(3,5-dichloro-4-hydroxyphenyl)methane, formed by incubation of 3,5-dichloro-4-hydroxybenzyl alcohol with 2,6-dichlorophenol- d_2 . The spectrum lacks ion peaks, illustrating the incorporation of the deuterium label of 2,6-dichlorophenol- d_2 into the product.

yield of the tetrachlorinated compound than was provided by the completely anaerobic incubations.

DISCUSSION

The environmental fate of CAM has not been the subject of any study so far. Nevertheless, they are important organohalogen compounds occurring in significant concentrations in the natural environment (10). In this study, the anaerobic biotransformation of the major fungal metabolite 3,5-dichloro-*p*-anisyl alcohol was evaluated. This metabolite is produced *de novo* up to concentrations of 108 mg per liter by *H. elongatum* (37). This is an important fungus occurring in wetlands, where anaerobic microniches are expected to adjoin the fungal colonies. The results taken as a whole indicate that 3,5-dichloro-*p*-anisyl alcohol is initially biotransformed in anaerobic environments via a demethylation reaction to 3,5-dichloro-4-hydroxybenzyl alcohol. This demethylated product is further converted via two routes. The first is a biotic route leading to the formation of 3,5-dichloro-4-hydroxybenzoate and 2,6-dichlorophenol. The second is an abiotic route leading to the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane. The proposed overall pathway is given in Fig. 4.

Demethylation. The key step in the biotransformation of 3,5-dichloro-*p*-anisyl alcohol is the demethylation of the methoxy moiety at the 4-position of the aromatic ring. The demethylation occurred without any lag phase in the case of WS-sludge and after 6 days of incubation, demethylation was complete, quantitatively yielding 3,5-dichloro-4-hydroxybenzyl alcohol as a product. The anaerobic bacteria of this sludge were precultivated on a substrate containing low-molecular-weight lignin compounds from wheat straw with similar methoxy moieties. This might explain the lack of any lag phase. However, it was remarkable that even in the incubations with VFA-sludge, which was grown on defined VFA for more than 3 years, demethylation started after 1 week and was completed after 2 weeks of incubation. Apparently, microorganisms with the capacity to demethylate methoxy aromatics were still present and viable in the VFA-sludge, since adaptation to the completely new substrate occurred rapidly.

There are some reports of demethylation of chlorinated

methoxy aromatics in the literature. Phenyl methyl ethers such as vanillic and syringic acids are examples of low-molecular-weight products derived from fungal lignin depolymerization (9, 22). These compounds were demethylated by acetogenic bacteria belonging to the genera *Acetobacterium*, *Eubacterium*, and *Clostridium* (4, 7, 8). *Clostridium thermoaceticum* as well as other anaerobic bacteria were shown to be able to grow by using the methoxyl group as an energy source (13, 29, 36). Also, chlorinated methoxy aromatics can be demethylated by anaerobic bacteria. Häggblom et al. (16) reported the anaerobic O demethylation of chlorinated guaiacols by the acetogenic bacteria *Acetobacterium woodii* and *Eubacterium limosum*. Anaerobic cell suspensions of both *E. limosum* and *A. woodii* were able to O demethylate di-, tri-, and tetrachloroguaiacols to the corresponding catechols, which accumulated and were not further metabolized. Liu and Jones (24) reported that 2,3- and

TABLE 1. Formation of bis(3,5-dichloro-4-hydroxyphenyl)methane from 3,5-dichloro-*p*-anisyl alcohol and 3,5-dichloro-4-hydroxybenzyl alcohol by wheat straw-grown methanogenic sludge after 7.5 weeks of incubation

Oxygen regimen ^a	Total concn (mM) of bis(3,5-dichloro-4-hydroxyphenyl)methane in substrate ^b :	
	3,5-Di-Cl- <i>p</i> -anisyl alcohol	3,5-Di-Cl-4-OH-benzyl alcohol
Living sludge		
–O ₂	0.043	0.040
–/+O ₂	0.075	0.064
+O ₂	ND ^c	0.094
Autoclaved sludge		
–O ₂	ND	0.150
–/+O ₂	ND	0.161
+O ₂	ND	0.178

^a –O₂, anaerobic conditions throughout; –/+O₂, anaerobic (first 10 days) and then aerobic (6 weeks) conditions; +O₂, aerobic conditions throughout.

^b Total concentration represents the sum of aqueous and solid bound concentrations.

^c ND, not detectable (detection limit, approximately 0.1 mg/liter [0.0003 mM]).

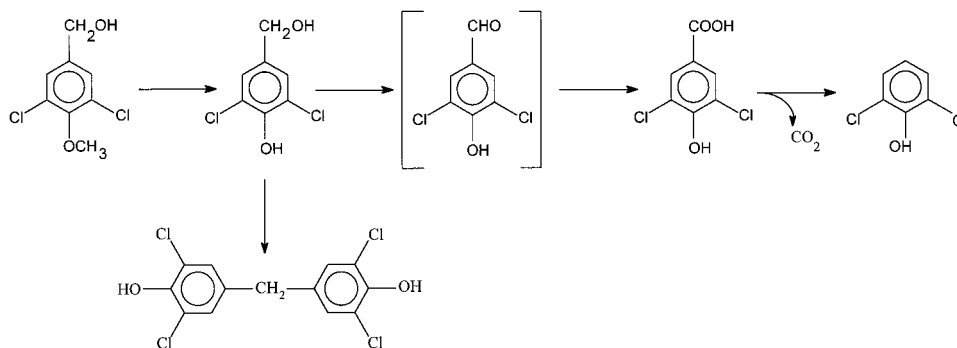


FIG. 4. Biotransformation pathway of 3,5-dichloro-*p*-anisyl alcohol by methanogenic sludge under anaerobic conditions and the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane.

3,5-dichloroanisole were initially demethylated by freshwater sediment slurries, producing 2,3- and 3,5-dichlorophenol.

The results from the literature therefore support the ubiquitous occurrence of acetogenic bacteria in anaerobic environments, which could catalyze the rapid demethylation of 3,5-dichloro-*p*-anisyl alcohol. Once the original fungal compound has been demethylated, 3,5-dichloro-4-hydroxybenzyl alcohol was converted further via two routes.

Route I. (i) Oxidation of benzyl alcohol. In route I, a biotic transformation of 3,5-dichloro-4-hydroxybenzyl alcohol occurred, leading to 3,5-dichloro-4-hydroxybenzoate and ultimately to 2,6-dichlorophenol. Similar anaerobic oxidations of the benzyl alcohol moiety to benzoate have been studied in relation to the anaerobic degradation of toluene. For the degradation of toluene in denitrifying bacteria, several pathways have been proposed. One of the proposed pathways, although disputed and contested, starts with an oxidation of the methyl group via benzyl alcohol and benzaldehyde to benzoate and further to benzoyl-coenzyme A (2, 23). Similar findings have also been reported under Fe(III) reducing and methanogenic conditions (14, 25). Methyl group oxidation by anaerobic bacteria has also been demonstrated with phenolic compounds such as *p*-cresol (5, 18). These studies showed that a *Achromobacter* sp. oxidized *p*-cresol to *p*-hydroxybenzyl alcohol with *p*-cresol methylhydroxylase, after which the latter compound was oxidized further to *p*-hydroxybenzoate.

Neilson et al. (31) studied the transformation of 3,5-dichloro-4-hydroxybenzaldehyde by metabolically stable anaerobic enrichment cultures. They reported that a small part of the 3,5-dichloro-4-hydroxybenzaldehyde was reduced to 3,5-dichloro-4-hydroxybenzyl alcohol, but that the majority was oxidized to 3,5-dichloro-4-hydroxybenzoate after 70 days of incubation. The results from Neilson et al. (31) indicate that perhaps 3,5-dichloro-4-hydroxybenzaldehyde was a transient intermediate in the oxidation of 3,5-dichloro-4-hydroxybenzyl alcohol to 3,5-dichloro-4-hydroxybenzoate, although the aldehyde was never detected in our studies.

(ii) Decarboxylation. The second step in the formation of 2,6-dichlorophenol is decarboxylation of 3,5-dichloro-4-hydroxybenzoate. Zhang and Wiegel (45) studied the anaerobic degradation of 3-chloro-4-hydroxybenzoate in freshwater sediments and found that the degradation of the substrate proceeds via either 2-chlorophenol or 4-hydroxybenzoate to phenol and subsequently to benzoate. In both possible degradation routes, a decarboxylation step is involved. In the study by Neilson et al. (31), 3,5-dichloro-4-hydroxybenzoate, which accumulated from the anaerobic oxidation of 3,5-dichloro-4-hydroxybenzaldehyde, was also decarboxylated and converted to

2,6-dichlorophenol, in a similar fashion to that observed in the incubations with WS-sludge in our study. A *Clostridium* species, which was able to transform 4-hydroxybenzoate and 3,4-dihydroxybenzoate and produced phenols as the final transformation product, was isolated and characterized (44). Later, Zhang et al. (46) isolated the amino acid-utilizing, hydroxybenzoate-decarboxylating bacterium *Clostridium hydroxybenzoicum* from methanogenic freshwater pond sediment, from which an oxygen-sensitive reversible 4-hydroxybenzoate decarboxylase was purified and characterized (17).

(iii) Dechlorination. Surprisingly, dechlorination of 2,6-dichlorophenol was not observed in these studies, although this compound is readily dechlorinated under methanogenic conditions. Possibly the incubation time of the experiments was too short for activating and building up a dehalogenating microbial population.

Route II. Formation of bis(3,5-dichloro-4-hydroxyphenyl)methane. In route II, an abiotic dimerization of 3,5-dichloro-4-hydroxybenzyl alcohol occurred, leading to the formation of the adduct bis(3,5-dichloro-4-hydroxyphenyl)methane. Based on the chemical synthesis of bis(3-chloro-4-hydroxyphenyl)methane by a condensation reaction of 3-chloro-4-hydroxybenzyl alcohol and 2-chlorophenol (6), it was expected that 2,6-dichlorophenol was involved in the formation of this adduct. Therefore, deuterium-labelled 2,6-dichlorophenol was added to 3,5-dichloro-4-hydroxybenzyl alcohol in the presence of living and autoclaved WS-sludge. However, it was observed that the label of 2,6-dichlorophenol was not incorporated into the adduct. This indicated that the adduct was most likely formed by a dimerization reaction of 3,5-dichloro-4-hydroxybenzyl alcohol. Next, it was observed that the concentration of adduct in incubations spiked with 3,5-dichloro-4-hydroxybenzyl alcohol were much higher in the bottles containing autoclaved sludge than in those containing living sludge, which indicated that formation of the adduct was an abiotic process. In the bottles containing living sludge, a competition between the biotic and abiotic routes probably occurred, leading to a mixture of biotransformation products and adduct. Although the dimerization reaction itself was due to an abiotic process, the biological process of 3,5-dichloro-*p*-anisyl alcohol demethylation was a prerequisite for adduct formation. The presence of oxygen prevented the formation of the adduct from 3,5-dichloro-*p*-anisyl alcohol in living sludge, probably because the acetogenic bacteria involved in the demethylation step are strict anaerobes (4). However, oxygen was stimulatory for the dimerization reaction in bottles spiked with 3,5-dichloro-4-hydroxybenzyl alcohol or if 3,5-dichloro-*p*-anisyl alcohol was first allowed to become demethylated by prior anaerobic incubation.

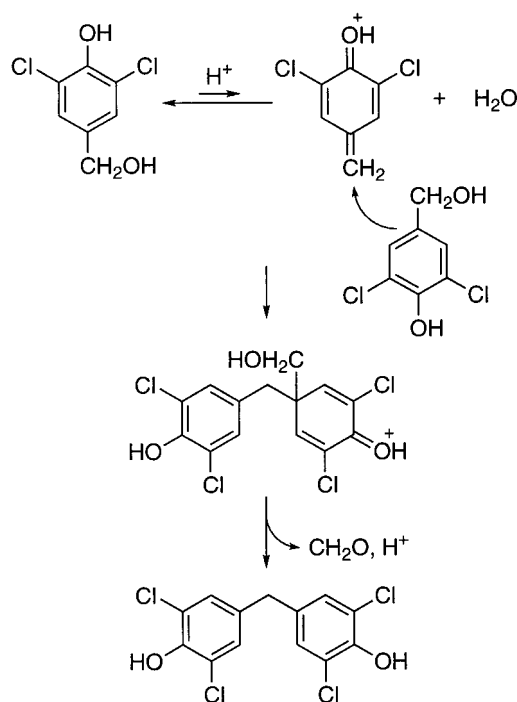


FIG. 5. Proposed reaction mechanism for the formation of bis(3,5-dichloro-4-hydroxyphenyl)methane by dimerization of 3,5-dichloro-4-hydroxybenzyl alcohol.

Although the actual mechanism is not known, the formation of the adduct most likely proceeds as outlined in Fig. 5. Under the influence of acids or metal salts present in the autoclaved sludge, heterolysis of 3,5-dichloro-4-hydroxybenzyl alcohol to a highly stabilized benzylic carbocation can take place. Attack of this electrophilic species on another molecule of 3,5-dichloro-4-hydroxybenzyl alcohol will then result in the formation of a dimeric intermediate which upon loss of formaldehyde gives the adduct. The adduct formation promoted by oxygen can also be explained by this process, but then radicals are involved. From the incubations, it is clear that the presence of autoclaved sludge was essential for the formation of adduct, since no dimerization occurred in sterile medium alone. It is not known which organic compound or metal in the sludge is responsible for the adduct formation.

Environmental implications. Assuming that bis(3,5-dichloro-4-hydroxyphenyl)methane is also formed in natural environments, its formation might have some important environmental implications. Wendel (41) tested the biological activity of the chemically produced adduct for use as pesticide and found that it had strong bactericidal activities against *Staphylococcus aureus*, *Streptomyces albus*, and *Streptococcus* sp. The tetrachlorinated compound also had antifungal activities (26). The adduct has a close resemblance to some widely used pesticides. Bis(5-chloro-2-hydroxyphenyl)methane (DCP) is a fungicide. This compound is used on wooden structures in industrial facilities and was reported to be very effective against many celluloytic fungi, which included *Penicillium*, *Aspergillus*, *Cladosporium*, and a *Trichoderma* sp. (28). Hexachlorophene is bis(3,4,6-trichloro-2-hydroxyphenyl)methane and is a pesticide with many applications in animal health care. Hexachlorophene was found to be very effective against *Fasciola* spp., which are parasitic flatworms living in the livers of cows and sheep and causing serious cattle diseases (33, 42). Hexachlorophene was demonstrated in cow milk at about 8 ng/ml in sam-

ples taken 24 h after oral administration of hexachlorophene to cows (19). Matsumura et al. (27) described that hexachlorophene hemolyzed washed human erythrocytes and inhibited acetylcholinesterase activities in erythrocyte membranes.

Bis(3,5-dichloro-4-hydroxyphenyl)methane also has a close resemblance to nonchlorinated compounds with a bisphenol-alkyl structure. Bisphenols, in particular 2,2-bis(4-hydroxyphenyl)propane (bisphenol A), are monomers of various plastics, including polycarbonates and epoxy resins, which are used in numerous consumer products. The release of bisphenol A from some of these materials into the food has recently been reported (32). Bisphenol A is an environmental estrogen (xenoestrogen) which belongs to a diverse group of estrogen-like chemicals that mimic estrogenic actions. Bisphenol A has estrogenic activity in vitro, probably caused by adverse effects on the neuroendocrine axis in susceptible human subpopulations (34). Gaido et al. (12) described how bisphenol A can interact directly with steroid hormone receptors in humans. In this way, bisphenol A and related compounds can bring disorder to the hormone balance of the human body, resulting in, among other effects, declining fertility.

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